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An Immunochemical Study of Structural and Evolutionary Relationships among Molluscan Octopine Dehydrogenases[1]

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ABSTRACT: Antisera produced against octopine dehydrogenases isolated from a gastropod and a cephalopod were used to investigate structural and evolutionary relationships of this enzyme in a range of mollusks. Antisera against octopine dehydrogenase of the blue-ringed octopus *Hapalochlaena maculosa* was most effective in inhibiting the enzyme from other octopods, followed by the enzymes of squids and cuttlefishes. Limited inhibition also occurred with octopine dehydrogenase of *Nautilus pompilius*, a representative of the most ancient group of living cephalopods. This antisera did not inhibit octopine dehydrogenases of gastropods or bivalves. Antisera against the enzyme of the gastropod *Strombus luhuanus* inhibited octopine dehydrogenases from other genera of the family Strombidae, but did not inhibit the enzyme from other families of gastropods or the enzymes from cephalopods or bivalves. It is concluded that the octopine dehydrogenases of cephalopods possess structural similarities and have diverged from a common ancestral gene. The structural and evolutionary relationships among gastropod octopine dehydrogenases and the relationships among octopine dehydrogenases from different molluscan classes remain unresolved.

OCTOPINE DEHYDROGENASE (EC 1.5.1.11) occurs in many mollusks and several other invertebrate phyla, and also in the crown gall tumors of plants where it is incorporated by a bacterial plasmid (Baldwin and England, this issue; Ellington 1979; Gäde 1980; Goldmann 1977; Hass et al. 1973; Regnouf and van Thoai 1970; Zammit and Newsholme 1976). The enzyme is unusual among dehydrogenases in being monomeric, with a molecular weight resembling the subunit size of most multimeric dehydrogenases (Baldwin and England, this issue; Fields, Baldwin, and Hochachka 1976*a*; Goldmann 1977; Olomucki et al. 1972).

In mollusk muscle, the octopine dehydrogenase reaction can replace lactate dehydrogenase in regenerating cytoplasmic NAD^+ during temporary anoxia associated with bursts of rapid locomotion (Baldwin, Lee, and England 1981; Gäde 1980). Octopine in plant crown gall tissue may serve as a carbon or nitrogen source for oncogenic agrobacteria (Drummond 1979).

The various invertebrate and plant octopine dehydrogenases possess structural and kinetic similarities; therefore, it is of interest to speculate on possible evolutionary relationships among these enzymes. In this study, an immunochemical approach has been taken to investigate structural and evolutionary relationships among the octopine dehydrogenases of cephalopod, gastropod, and bivalve mollusks.

MATERIALS AND METHODS

Experimental Animals

Blue-ringed octopuses, *Hapalochlaena maculosa*, were captured by dredging on

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mussel beds in Port Phillip Bay, Victoria, Australia. *Strombus luhuanus* were collected on the reef flat at Heron Island, Queensland, Australia. Muscles from these animals were frozen in liquid nitrogen immediately after death and stored at −20°C for up to 6 months without loss of octopine dehydrogenase activity. This provided the material from which cephalopod and gastropod octopine dehydrogenases were isolated for antibody production. *Pecten alba* came from Port Phillip Bay, while other mollusks used in the study were collected in the Republic of the Philippines during the *Alpha Helix* expedition.

Isolation of Octopine Dehydrogenases for Antibody Production

Frozen mantle and arm muscle of the blue-ringed octopus was homogenized in 10 vol of ice-cold buffer [25 mM Tris–HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM 1,4-dithioerythritol (DTE), 10% glycerol, pH 8.5] using a Sorvall omni-mixer. The homogenate was centrifuged at 20,000 × *g* for 30 min at 4°C, and the pellet discarded. The portion of the supernatant precipitating between 35 and 75% ammonium sulfate saturation was collected by centrifugation, dissolved in a small volume of the homogenizing buffer, and dialyzed against a large volume of this buffer. The dialyzed sample was applied to a *O*-(diethylaminoethyl) (DEAE) cellulose column (Whatman DE22) equilibrated with the homogenizing buffer, and octopine dehydrogenase activity was washed through with this buffer. Pooled fractions from the DEAE column were concentrated by ammonium sulfate precipitation and dialyzed against 10 mM sodium phosphate buffer, pH 6.6, containing 0.1 mM EDTA, 0.1 mM DTE, and 10% glycerol. The dialyzed sample was loaded onto a *O*-(carboxymethyl) (CM) cellulose column (Whatman CM23) equilibrated with the dialysis buffer. Octopine dehydrogenase activity was eluted by washing with the equilibration buffer. Fractions with the highest specific activity were concentrated by membrane filtration (Amicon Diaflo, PM10) and further purified by gel filtration on Sephadex G100 equilibrated with 50 mM Tris–HCl buffer, pH 7.6, containing 0.1 mM EDTA, 0.1 mM DTE, and 10% glycerol. The most active fractions were pooled, concentrated by membrane filtration, and stored at 4°C in 80% saturated ammonium sulfate.

Octopine dehydrogenase was purified from the pedal retractor muscle of *Strombus luhuanus* as described by Baldwin and England (this issue).

Production of Antisera

Antisera against octopine dehydrogenases isolated from the blue-ringed octopus and *Strombus luhuanus* were produced in adult New Zealand white rabbits. On day 0, 3 mg of protein in 1 ml of 20 mM sodium phosphate/50 mM sodium chloride buffer, pH 7.4, was emulsified with an equal volume of Freund's complete adjuvant[3] and injected subcutaneously at multiple sites in the hind leg. This procedure was repeated twice at intervals of 7 days. A final injection of 1 mg of protein in 1.5 ml of buffer was administered on day 21. Blood was collected from the marginal ear vein each week from day 28. Serum from the clotted blood was centrifuged and stored at −20°C in 0.5-ml aliquots. A sample of control serum was collected from each rabbit prior to the initial injection of octopine dehydrogenase.

Preparation of Octopine Dehydrogenase Extracts for Immunochemical Titration

Mantle muscle from octopods, squids, and cuttlefishes; spadix of *Nautilus*; pedal retractor muscle of gastropods; and adductor muscles of bivalves were homogenized in 10 vol of ice-cold phosphate buffer (50 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM DTE, 10% glycerol, pH 7.0) and centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatants were stored at −20°C and used as a source of octopine dehydrogenase for immunochemical titrations.

[3] Mineral oil 85% (v/v), mannide monooleate 15% (v/v), *M. tuberculosis* (heat-killed) 0.5 mg/ml.

Electrophoresis

Octopine dehydrogenase extracts were examined by electrophoresis on cellulose acetate gels (Cellogel, Chemtron, Milano) using 75 mM Tris–citrate buffer, pH 7.5. The gels were stained for octopine dehydrogenase activity as described by Fields et al. (1976*b*).

Assay of Octopine Dehydrogenase Activity

Octopine dehydrogenase activity was determined at 340 nm with a Zeiss DM 4 recording spectrophotometer. The cell temperature was maintained at 25°C with a circulating water bath. Assays were carried out with from 5 to 25 μl of sample in a total volume of 1 ml. The reaction mixture, which was selected to give maximum activity with most of the tissue extracts examined, contained 5 mM sodium pyruvate, 20 mM arginine, and 0.2 mM NADH in 50 mM sodium phosphate buffer, pH 7.0. The rate measured with this assay mixture is equal to the sum of the activities of both octopine and lactate dehydrogenases, and lactate dehydrogenase was potentially present in both the mollusk muscle extracts and the rabbit serum. Octopine dehydrogenase values were corrected by subtracting the rate due to lactate dehydrogenase alone when arginine was omitted from the assay. One unit of enzyme activity was defined as the amount required to oxidize 1 μmole NADH/min.

Immunochemical Titration of Octopine Dehydrogenase in Mollusk Muscle Extracts

Appropriate volumes of muscle extract containing 0.5 IU of octopine dehydrogenase activity were mixed with varying amounts of antiserum, control serum, and buffer (50 mM sodium phosphate, 0.1 mM EDTA, 0.1 mM DTE, 10% glycerol, pH 7.0) such that the serum concentration was kept constant in a final volume of 100 μl. The samples were incubated for 1 hr at 25°C, 16 hr at 4°C, and then centrifuged at 20,000 × *g* for 30 min at 4°C. The supernatants were assayed for octopine dehydrogenase activity to determine the percentage inhibition of the enzyme by the antiserum.

RESULTS AND DISCUSSION

The inhibition curves (Figures 1, 2) show the effects of increasing amounts of antiserum on the activity of octopine dehydrogenases from various mollusks. The relationship between the percentage inhibition and the volume of antiserum added is linear only in the region of the curve where enzyme inhibition is less than about 40%. To obtain numerical inhibition values for comparing the different enzymes tested, the initial linear portion of each curve was extrapolated to the bottom axis. This inhibition value corresponds to the number of microliters of antiserum that would have inhibited one unit of octopine dehydrogenase activity if the relationship between percentage inhibition and volume of antiserum had remained linear (Jokay and Toth 1966, Mochizuki and Hori 1980).

Electrophoresis gave a single major anodally migrating band of octopine dehydrogenase activity for each of the cephalopod and gastropod muscle extracts in which enzyme activity was inhibited by antisera. It is assumed that the inhibition curves reflect interactions between the antiserum and a single molecular form of octopine dehydrogenase.

Results obtained with antiserum prepared against octopine dehydrogenase of the blue-ringed octopus are shown in Figure 1 and Table 1. This antiserum was most effective in inhibiting the enzymes from other octopods, followed by the enzymes of squids and cuttlefishes. More limited inhibition occurred with octopine dehydrogenase from the nautiloid, *Nautilus pompilius*, a representative of the most ancient group of living cephalopods. The relative inhibition potencies reflect the generally accepted phylogenetic relationships among the subclasses and orders of cephalopods examined (Donovan 1977). This antiserum did not inhibit octopine dehydrogenases from the five species of gastropods and two species of bivalves tested.

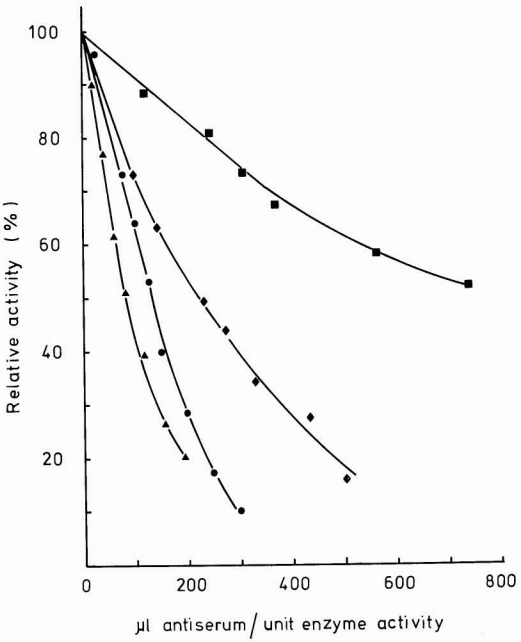

FIGURE 1. Inhibition curves of cephalopod octopine dehydrogenases with antiserum produced against octopine dehydrogenase of the blue-ringed octopus. LEGEND: ▲, *Octopus macropus*; ●, *Thysanoteuthis rhombus*; ♦, *Sepia bandensis*; ■, *Nautilus pompilius*.

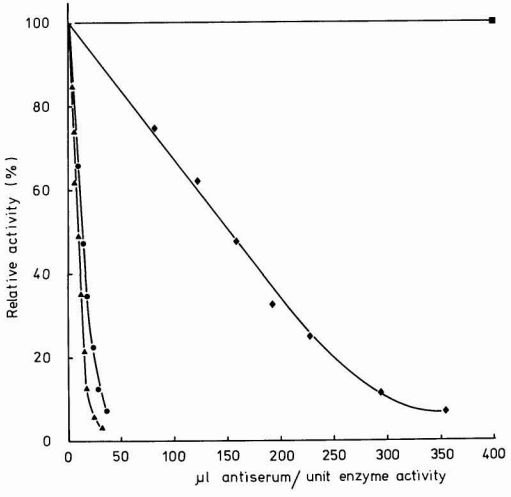

FIGURE 2. Inhibition curves of gastropod octopine dehydrogenases with antiserum produced against octopine dehydrogenase of *Strombus luhuanus*. LEGEND: ▲, *Strombus canarium*; ●, *Lambis chiragra*; ♦, *Tibia martinii*; ■, *Cerithium nodulosium*.

TABLE 1

IMMUNOCHEMICAL TITRATION OF OCTOPINE DEHYDROGENASE ACTIVITY IN MOLLUSK MUSCLE EXTRACTS WITH ANTISERUM PRODUCED AGAINST OCTOPINE DEHYDROGENASE OF THE BLUE-RINGED OCTOPUS, *Hapalochlaena maculosa*

SPECIES	INHIBITION VALUE*	RELATIVE INHIBITION POTENCY†
Octopods		
Hapalochlaena maculosa	65	1
O. membranaceus	80	0.813
O. macropus	160	0.406
O. horridus	180	0.361
Squids		
Thysanoteuthis rhombus	265	0.245
Symplectoteuthis oualaniensis	300	0.217
Sepioteuthis lessoniana	310	0.210
Cuttlefishes		
Sepia latimanus	320	0.203
S. bandensis	390	0.167
Nautiloid		
Nautilus pompilius	1,200	0.054
Gastropods		
Strombus sinuatus	N.I.	
Lambis scorpius	N.I.	
Tibia	N.I.	
Nassarius coronatus	N.I.	
Cerithium nodulosium	N.I.	
Bivalves		
Pecten alba	N.I.	
Periglypta reticulata	N.I.	

*Values in microliters of serum required to inhibit 1 unit of enzyme activity. N.I. indicates no inhibition.

†Inhibition value relative to *Hapalochlaena maculosa* octopine dehydrogenase.

Antiserum produced against octopine dehydrogenase of the gastropod *Strombus luhuanus* (Figure 2, Table 2) inhibited the enzyme from the three genera of the family Strombidae tested. The results did not clearly distinguish between octopine dehydrogenases of *Strombus* and *Lambis*. However, the relative inhibition potency value obtained for octopine dehydrogenase of *Tibia* shows that this enzyme is antigenically quite distinct from the enzymes of the other two genera. This antiserum had no inhibitory effect on the octopine dehydrogenases from the other five families of gastropods tested, or on the enzymes from cephalopods or bivalves.

Estimating structural and evolutionary re-

TABLE 2

IMMUNOCHEMICAL TITRATION OF OCTOPINE DEHYDROGENASE ACTIVITY IN MOLLUSK MUSCLE EXTRACTS WITH ANTISERUM PRODUCED AGAINST OCTOPINE DEHYDROGENASE OF *Strombus luhuanus*

SPECIES	INHIBITION VALUE*	RELATIVE INHIBITION POTENCY†
Gastropods		
Family Strombidae, *Strombus luhuanus*	20	1
S. canarium	21	0.952
S. labiatus	21	0.952
S. aurisdianae	24	0.833
S. sinuatus	25	0.800
S. lentiginosus	27	0.741
Lambis scorpius	22	0.909
L. lambis	22	0.909
L. chiragra	26	0.769
L. millepeda	28	0.714
Tibia martinii	300	0.067
Family Buccinidae, *Cantharus undosus*	N.I.	
Family Bursidae, *Bursa* sp.	N.I.	
Family Muricidae, *Murex tribulus*	N.I.	
Family Nassariidae, *Nassarius coronatus*	N.I.	
Family Cerithiidae, *Cerithium nodulosium*	N.I.	
Cephalopods		
Hapalochlaena maculosa	N.I.	
Sepioteuthis lessoniana	N.I.	
Sepia latimanus	N.I.	
Nautilus pompilius	N.I.	
Bivalves		
Pecten alba	N.I.	
Periglypta reticulata	N.I.	

*Values in microliters of serum required to inhibit 1 unit of enzyme activity. N.I. indicates no inhibition.
† Inhibition value relative to *Strombus luhuanus* octopine dehydrogenase.

lationships among enzymes by immunochemical inhibition has proved successful with a range of enzymes from both vertebrates and invertebrates (Matsuoka and Hori 1980, Mochizuki and Hori 1980, Sado and Hori 1978). A major advantage of this method when comparing enzymes from a large number of organisms is that it requires only isolation of the enzymes used to produce antisera. Even if these enzymes are not purified to homogeneity, the specificity of the enzyme assay system should avoid problems associated with interactions of the antiserum with other proteins in the tissue homogenates tested. However, difficulties may arise with this technique because differences in relative inhibition potency among homologous enzymes may reflect not only structural differences, but also differences in turnover number (amount of substrate transformed per unit time per enzyme molecule). For example, in a group of homologous enzymes containing identical antigenic sites but differing in turnover number, enzymes with highest turnover numbers will appear to be most susceptible to inhibition. This is because the ratio between the volume of antiserum added and the number of enzyme molecules in the assay system is greater per unit of enzyme activity. These potential problems associated with differences in turnover number do not appear to be significant in the present study. The ordering of relative inhibition potencies obtained for both the cephalopod and gastropod octopine dehydrogenases follow the accepted phylogenetic relationships among the animals tested. This suggests that the accumulation of structural changes in the enzyme with time

has not been masked by differences in turnover number.

The results obtained in this study show that the octopine dehydrogenases of octopods, squids, cuttlefishes, and nautiloids are structurally similar in that they share common antigenic determinants. Presumably, these enzymes are homologous, having diverged from a common ancestral gene that was present in the class Cephalopoda prior to the separation of the subclasses Nautiloidea and Coleoidea. Evidence from the fossil record indicates that this separation had already occurred by the early Carboniferous (Donovan 1977, Stasek 1972).

More limited information was obtained on the relationships among gastropod octopine dehydrogenases. The enzymes from the three genera of the family Strombidae clearly possess structural similarities. However, little can be said of the structural relationships among the octopine dehydrogenases of the Strombidae and the octopine dehydrogenases from other gastropod families, as the latter were not inhibited by antisera produced against the *Strombus luhuanus* enzyme.

Similarly, failure of the antisera produced against cephalopod or gastropod octopine dehydrogenases to inhibit the enzyme from other molluscan classes leaves unresolved the structural and evolutionary relationships among octopine dehydrogenases of cephalopods, gastropods, and bivalves. The extant molluscan classes are considered to have diverged from some common ancestral group during the Precambrian, but evidence of these events is lacking from the fossil record. Stasek (1972:3) has proposed that the stem groups that gave rise to the antecedents of mollusks, annelids, and arthropods "could have been placed in an expanded concept of the turbellarian Platyhelminthes or in a single, now defunct, phylum that would have included the extant Nemertinea." It is of interest when considering the evolutionary relationships of molluscan octopine dehydrogenases that octopine has been found in the nemerteans *Cerebratulus occidentalis* and *Lineus pictifrons* (Robin 1964), and that octopine dehydrogenase occurs in the sipunculid *Sipunculus nudus* (van Thoai and Robin 1959).

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